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(54) Title: IMMUNOREACTIVE COMPOUND

(57) Abstract

Novel immunoreactive compounds are provided which comprise one or more antigen-binding fragments of IgM or IgA coupled to a carrier molecule, such as HSA, an enzyme or a synthetic polypeptide of low immunogenicity. By coupling these fragments to the carrier their relatively low antigen affinity is restored to a level comparable with the affinity level of native IgM or IgA.

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Immunoreactive compound

The present invention relates to an immunoreactive compound and a pharmaceutical preparation comprising the same.

Such an immunoreactive compound can particularly be used in immunotherapy and in diagnosis.

Immunotherapy is one of the promising possibilities to fight a number of diseases. The principle of immunotherapy itself is old. It comprises a targeting moiety which delivers an active substance to the immediate vicinity of the target.

Thus it can be used to kill, or optionally stimulate a certain group of cells which share a site for which a targeting moiety is available. Ligand-receptor interactions or antibody-antigen interactions are suitable couples of targeting moiety and target, but others can of course be envisioned by the person skilled in the art.

A probably more elegant way of immunotherapy are the so called pretargeting strategies. These include, but are not limited to, prodrug activation, whereby an enzyme is coupled to a targeting moiety, which is administered before or together with a prodrug which is less toxic than its parent drug and which enzyme converts the prodrug into the parent drug at the target site.

For anti-tumour therapy and tumour localization, and for cancer diagnosis generally use is made of antibodies coupled to a label. In anti-tumour therapy such a label can be e.g. a toxic compound such as adriamycin, verrucarin, calicheamycin, mitomycin, ricin a, or any other suitable toxic compound, or an isotope or, as described above an enzyme.

The antibodies generally are targeted against a particular antigen of the tumour. The antibodies used for this purpose in most cases are monoclonal antibodies of murine origin. Murine monoclonal antibodies are easy to obtain according to well established methods, and against virtually any antigen.

Nevertheless these antibodies have several drawbacks. If tumour material is administered to mice these develop antibodies against almost any antigen of the tumour material, including the normal antigens of these cells. In this way it is difficult to obtain antibodies specific for tumour cells only. Hence, murine antibodies may be directed to epitopes that are not tumour-specific according to the human immune repertoire. Furthermore the use of murine antibodies is hampered by their inherent immunogenicity in humans. A solution has been sought in the use of antibody fragments mainly containing the antigen binding domain of the murine antibody, which may overcome the second, but certainly not the first problem.

A more ideal solution resides in the use of human anti-tumour antibodies. Human anti-tumour antibodies can suitably be obtained according to the method described in EP 0151030. A problem is, however, that this method mainly yields immunoglobulins of the IgM and/or IgA type. These are in fact pentameric or dimeric, i.e. they are composed of five or two monomers interconnected via S-S bridges, whereas each of these IgM/IgA monomers is composed of two heavy and two light chains and contains two antigen binding sites. Each of these monomers roughly equals an IgG molecule in size. Hence a complete IgM molecule is about five times the size of an intact IgG molecule. Most IgM's are characterized by antigen-affinities which are at the low end of the IgG affinity range.

The relatively large dimensions of the IgM molecules make them less suitable for in vivo use for immunotherapy and for tumour imaging; it takes a relatively long time for them to reach the target site and the clearance of unbound IgM takes at least five times longer than for IgG.The same goes for IgA's, though not in the order of magnitude as with IgM's.

A straightforward solution looks to be the fragmentation of the IgM pentamer or the IgA dimer into its monomers. However, it has been reported that the antigen-affinity of the IgM/IgA monomers is dramatically lower than the affinity of intact IgM/IgA; the difference amounts to at least about a hundred to thousand-fold. This low affinity makes the IgM/IgA monomer unsuitable for therapeutic and diagnostic application. A similar lowering of affinity was found for enzymatically obtained fragments of IgM and IgA.

The present invention is concerned with the restoration of the antigen affinity of fragments of IgM. According to the present invention the affinity of antigen binding IgM or IgA fragments can be restored by coupling them to at least one polypeptide.

Such a polypeptide can advantageously be a human protein such as human serum albumin, or a (human) enzyme, or it can be a synthetic polypeptide with a low immunogenicity such a poly-L-glutamic acid or poly-L-lysine. The IgM or IgA fragment either can be an IgM or an IgA monomer, which can be obtained by reducing the S-S bonds between the monomers, or can be an antigen binding fragment obtained after enzymatic cleavage of the IgM or the IgA, e.g. by use of pepsin or papain. Digestion with pepsin delivers an antibody fragment generally indicated as $F(ab')_2$ which in turn is composed of two antigen bindings parts interconnected by S-S bonds. Reduction of these bonds yields two F(ab') fragments.

Both IgM or IgA monomers and F(ab') fragments ideally can be bound to the polypeptide(s) via their sulphur atom. However, binding of the IgM fragment to the polypeptide can be by any other suitable bond, as long as the antigen binding characteristics are not hampered. In this respect it is also convenient to establish a binding via glycosyl groups if present at the constant region of the IgM or IgA fragment.

The bond between the IgM or IgA fragment and the polypeptide can either be a direct link or an indirect link via a linking group and/or a spacer.

The bond between the IgM or IgA fragment and the polypeptide can be established by making available on both components a group suitable for linking, optionally reacting either or both linking groups with a linker and/or spacer, and thereafter reacting the components to form the desired immunoreactive compound.

Optionally the polypeptide can be labelled with one or more therapeutically or diagnostically useful groups prior to or after the coupling to the IgM or IgA fragment. Suitable therapeutically useful groups are e.g. cytotoxic drugs, (optionally chelated) radioactive atoms, or enzymes for the conversion of prodrugs into active drugs.

However, if the polypeptide is an enzyme itself, which is able to convert a prodrug into a drug at the target site, there is an additional advantage, because the size of the immunoreactive compound plays an important role in its applicability. Suitable diagnostically useful groups are e.g. (optionally chelated) radioactive atoms. The IgM or IgA fragment advantageously is obtained from human IgM or IgA. This IgM or IgA is directed against an antigen specific for, or derived from the tumour, which may be found either in or on or outside the tumour cells.

Example 1

A. Preparation of immunoreactive IgM monomers.

Human monoclonal IgM antibodies 16-88 against tumour associated epitopes occurring in colorectal cancer were converted into monomers by reduction with cysteine.

IgM (3-5 mg/ml) was incubated in 10 mmol/l cysteine in PBS (6.7 mmol/l K/Na phosphate buffer PH 6.5; 0.13 mol/l NaCl) for 3 h at 37 ^{o}C. Buffers were saturated with nitrogen and the reaction vessel was closed gas-tight. After incubation, the reaction mixture was chromatographed on Sephadex G25, equilibrated with 1 mmol/l cysteine in PBS. Monomers were precipitated with $(NH_4)_2SO_4$ at 50% saturation and dissolved in a minimal volume of 1 mmol/l cysteine in PBS PH 7.5. The monomer solution was applied on a Fractogel TSK HW 55 (S) column equilibrated in 1 mmol/l cysteine in PBS PH 7.5. The bed volume of the column was 45 times that of the volume applied and the elution rate was 0.06 bed volume/h. Monomers were usually eluted at a Kav=0.55-0.60, in a predominant A_{280} peak. They were precipitated by $(NH_4)_2SO_4$ at 50% saturation. After dissolving the precipitate in 0.1 mol/l Sodium phosphate, 0.1 mol/l NaCl, 5 mmol/l EDTA, 1 mmol/l cysteine PH 7.5, residual ammonium sulphate was removed by gel filtration on Sephadex G25 equilibrated in the EDTA containing PBS buffer mentioned above.

Solid DTNB (dithionitrobenzoic acid, Ellman's reagent) was added to the desalted monomer-containing fraction to a final concentration of 20 mmol/l and after gently shaking the reaction mixture was incubated for 3 h at ambient temperature.

Excess reagent and low molecular weight reaction products were removed by gel filtration on Sephadex G25 in 0.1 mol/l sodium phosphate; 0.1mol/lNaCl; 5mmol/l EDTA PH 7.5 (Solution A).

B. Reduction of HSA(-DTPA).

HSA(-DTPA) was dissolved to a concentration of 5-10 mg/ml in 0.1 mol/l sodium phosphate; 0.1 mol/l NaCl; 5 mmol/l EDTA PH 7.5. To this solution, DTT (dithio-threitol) was added to a final concentration of 20 mmol/l and incubation was performed for 30 min. at ambient temperature. The reaction mixture was then chromatographed on Sephadex G25 equilibrated in the EDTA/PBS PH 7.5 mentioned above in order to remove excess of reducing agent and low molecular weight reaction products (Solution B).

C. Preparation of IgM-HSA(-DTPA) immunoconjugates.

Immediately after reduction of HSA(-DTPA), solution A (containing activated monomers) and solution B (containing reduced HSA) were mixed, the monomers/HSA(-DTPA) mass ratio being around 0.5. Incubation was performed overnight at ambient temperature.

After completion of the conjugation reaction, non-conjugated HSA(-DTPA) was removed by $(NH_4)_2SO_4$ precipitation at 50% saturation. Conjugate and non-conjugated monomers were precipitated, whereas HSA(-DTPA) remains in solution. The precipitate was washed several times with 50 % saturated $(NH_4)_2SO_4$ and was dissolved in a minimal volume of 0.1 mol/l sodium phosphate, 5 mmol/l EDTA PH 7.5. The solution was chromatographed on Sephadex G25 equilibrated in the EDTA phosphate buffer (buffer A) (devoid of NaCl !!). The protein-containing fraction was then applied on a

Q-Sepharose (Fast Flow) column equilibrated in buffer A in order to separate non-conjugated monomers from the immunoconjugate by anion-exchange chromatography. After application of the sample, the Q-Sepharose column was washed with buffer A until the A_{280} had returned to baseline level. Proteins retained by the column were eluted by stepwise increasing the NaCl concentration from 0 - 0.6 mol/l NaCl.

The bulk of the non-conjugated monomers passed the column in the fall-trough fraction, whereas the immunoconjugate was retained because of the acidic character of HSA(-DTPA) and was eluted at 0.3-0.4 mol/l NaCl in buffer A. After desalting the immunoconjugate on Sephadex G25, the final preparation was sterilised by filtration through a 0.20 μm membrane and stored in small aliquots at 4 ^{o}C until use.

Example 2

Preparation of IgM Fab'-HSA(-DTPA) conjugates.

IgM was digested with pepsin according to the method of Putnam. Briefly, whole IgM at a concentration of 2-5 mg/ml in 0.1 mol/l sodium acetate buffer PH 4.0 was incubated with pepsin (0.08-0.2 mg/ml) for 8 h at 4 ^{o}C. The reaction mixture was chromatographed on Fractogel HW55S equilibrated in buffer A in order to purify the $F(ab')_2$ fragments formed from whole, undigested IgM and low molecular weight fragments. In this way, chromatographically pure $F(ab')_2$ was isolated at a 40-60% yield.

$F(ab')_2$ was reduced with DTT and coupled to HSA(-DTPA) in the way described for the monomers. Purification of F(ab')-HSA(-DTPA) was achieved by anion exchange chromatography on Q-sepharose and gel filtration on Fractogel as described above. The final prep was sterilised by filtration and stored at 4 ^{o}C.

Example 3

Immunoreactivity of IgM, IgM monomers, IgM F(ab')$_2$ fragments, IgM monomer-HSA(-DTPA) and IgM Fab'-HSA(-DTPA)-immunoconjugates.

Immunoreactivity was determined by either an antigen binding assay or competitive EIA.

In the antigen binding assay (Dot-blot EIA) dilution series of the samples to be tested are transferred into Immobilon membranes in a Biorad Trans blot apparatus. Excess protein binding sites are blocked with 5% Skim milk and the blots are then incubated with peroxidase-labelled antigen in PBS buffer. After 2 h of incubation at ambient temperature. The antigen-containing solution is discarded, the blot is washed three times with PBS-Tween and the enzyme borinol is detected with a substrate solution containing 2 mmol/l hydrogen peroxide and 0.6 mg/ml diaminobenzidine, 0.6 mg/ml $CoCl_2$ as hydrogen donor. Violet coloured spots become visible after 5 min. of incubation. The colour intensity is measured by scanning in a Biorad gel scanner.

In the competitive EIA, dilution series to be tested are incubated with a given amount of peroxidase-labelled whole IgM for 3 h at ambient temperature in an microtitre plate coated with 0.1 µg/ml antigen solution. After incubation, the contents of the wells is discarded and the plates are washed three times with PBS-Tween buffer. Enzyme activity is detected with a substrate solution containing tetramethylbenzidine as hydrogen donor. The enzyme

reaction was stopped with 2 mol/l H_2SO_4 and the absorbance was read at 450 nm.

Both assays give comparative results with respect to immunoreactivity of IgM, IgM fragments and immunoconjugates.

Purified monomers have a specific immunoreactivity (=IR per mass) of 0.001-0.05 times that of the untreated, whole IgM, whereas $F(ab')_2$ fragments are not immunoreactive in the assays applied. However purified HSA(-DTPA) conjugates of monomers exhibit an immunoreactivity identical to that of whole IgM and F(ab')-HSA(-DTPA) conjugates show some immunoreactivity comparable to that of purified monomers.

In conclusion, covalent attachment of a (carrier) protein, e.g. through disulphide or thioether bridges, is able to restore the immunoreactivity of IgM monomers or fragments considerably, even to full extent in case of monomers.

Example 4

Preparation of Enzyme conjugate

(Enzyme-)conjugates could be prepared in two ways:

A: By direct conjugation to SPDP-activated enzyms

B: By activition of the monomer with DTNB, followed by reaction with the enzyme, having free -SH groups.

4.1. Method A

To the enzyme (10 mg/ml in 0,1 M Na_2HPO_4/NaH_2PO_4 ph 7,5 ; 0,1 M naCl) 1/10 volume of 40 mM SPDP (disolved in absolute ethanol) was added and incubation was performed for 30 min. at room temperature in the dark. The reaction mixture was chromatographed on a Sephadex G-25 (M) column equilibrated with the same buffer to remove unreacted SPDP. The SPDP activated enzyme was added to the IgM-monomer (which was just before addition also chromatiographed over Sephadex G-25 (M) equilibanted in this buffer) in a ratio of 1 : 1 (w/w).

The reaction mixture was incubated for 16 hours at room temperature in the dark. The conjugate was recovered by addition of an equal volume of 100 % satured ammonium sulph. The precipitate was washed three times with 50 % saturated ammonium sulph, before being dissolved in an appropiate buffer.

(Remark: These final steps could be performed using HRP as an enzyme, for other enzymes other purification methods may be necessary).

4.2. Method B

Activation of the enzyme:

First the enzyme was activated by SPDP us described in method A. The activated enzyme was chromatiografied on a Sephadex G-25 (M) column equilibrated in 0,1 M NaAc pH 4,5; 0,1 M NaCl. To the enzyme containing fractions 1/20 volume of 1M DTT was added and incubation was performed for at least 30 minutes at room temperature in the dark.

Just before coupling this mixture was chromatografied over a Sephades G-25 (M) procedure for the preparation of IgM-monomer-enzyme conjugates.

4.3. Monomerisation

(Human)IgM was monomerised by incubation at 37°C for 3 h in the following solution (saturated with N_2).
5 mM Na^2HPO_4/NaH_2PO_4 pH 6,5
65 mM NaCl
0,1 g/L NaN_3 10 mM Cysteïne
0,5 U papaïne/g IgM
1-10 g/h IgM

After this incubation an equal volume of 100 % saturated ammonium sulphate solution was added.

After standing at least 2 hours at 4°C, the precipitate was recovered (after centrifugation) and dissolved in:
50 mM Tris.HCl pH 8
140 mM NaCl
1 mM Cysteïne

To remove residual ammonium sulphate this resolution was chromatographed over a Sephades G-25 (M) columns into the same buffer.

4.4. Purification of IgM-momomer

The above mentioned solution was chromatographed over a Fraktogel HW-55 (S) column (, equilibrated into the same buffer).

The fractions containing the monomerie IgM, (,usually the second peak) were pooled and concentrated by addition of an equal volume of 100 % saturated ammonium sulphate in a 1 mM Cysteïne-solution. The precipitate was collected and used for conjugation.

4.5 Activation of monomerie IgM

The monomerie IgM was chromatographed over Sephadex G-25 (M) into :

50 mM Tris-HCL pH 8

140 mM NaCl

1 mM Cysteine

To this solution 15 mg DTNB/ml solution were added (in a solid form) and made to dissolve. The reaction mixture was incubated for 16 hours at room temperature in the dark. Unreacted DTNB was removed by chromatography over Sephadex G-25 (M), equilibrated in 0,1 M Na_2HPO_4/NaH_2PO_4 pH 7,5; 0,1 M NaCl.

The degree of activation of the monomer with TNB could be determined in the following manner:

$$\text{conc. of monomer} = \frac{E\ 280}{1{,}45 \times Mw\ (=180.000)} \quad (M)\ (=A)$$

$$\text{conc. of TNB} = \frac{E\ 330}{9217}\ (M)\ (=B)$$

$$\text{TNB/monomer} = B/A$$

Conjugation: The actived HRP and the activated monomeric IgM were added to each order in a ratio 1:1 (w/w). Incubation was performed for 16 hours at room temperature in the dark. After this period the conjugate was purified as described in method A.

4.6

The immuno reactivity of the enzyme conjugates was determined by incubation of the conjugate with a microtiter plate coated with crude antigen mixture of a tumour cell line expressing antigens that are recognized by Monoclonal antibodies 16.88 and 81AV78 (shown in fig. 1). Mab 16.88 recognices specifically a tumour-associated epitope on cytokeratins, whereas Mab

81MV78 reacts with a tumour-associated antigen at the surface of the cells.

However a conjugate of an indifferent antibody a myecloma IgM with no reactivity towards tumour cells or cell lines, did not bind to the crude antigen preparation.

Mycloma is an antibody (IgM) recognizing an antigen not present in the mixture of crude antigens.

CLAIMS

1. Immunoreactive compound comprising an antigen binding fragment of IgM or IgA coupled to at least one polypeptide.

2. Immunoreactive compound according to claim 1 characterized in that the fragment is of human IgM or IgA.

3. Immunoreactive compound according to claim 1-2, characterized in that the IgM or IgA is directed against a tumour-associated antigen.

4. Immunoreactive compound according to claim 1-3, characterized in that the polypeptide is a human protein.

5. Immunoreactive compound according to claim 1-4, characterized in that the polypeptide is human serum albumin or a fragment thereof.

6. Immunoreactive compound according to claim 1-4, characterized in that the polypeptide is an enzyme.

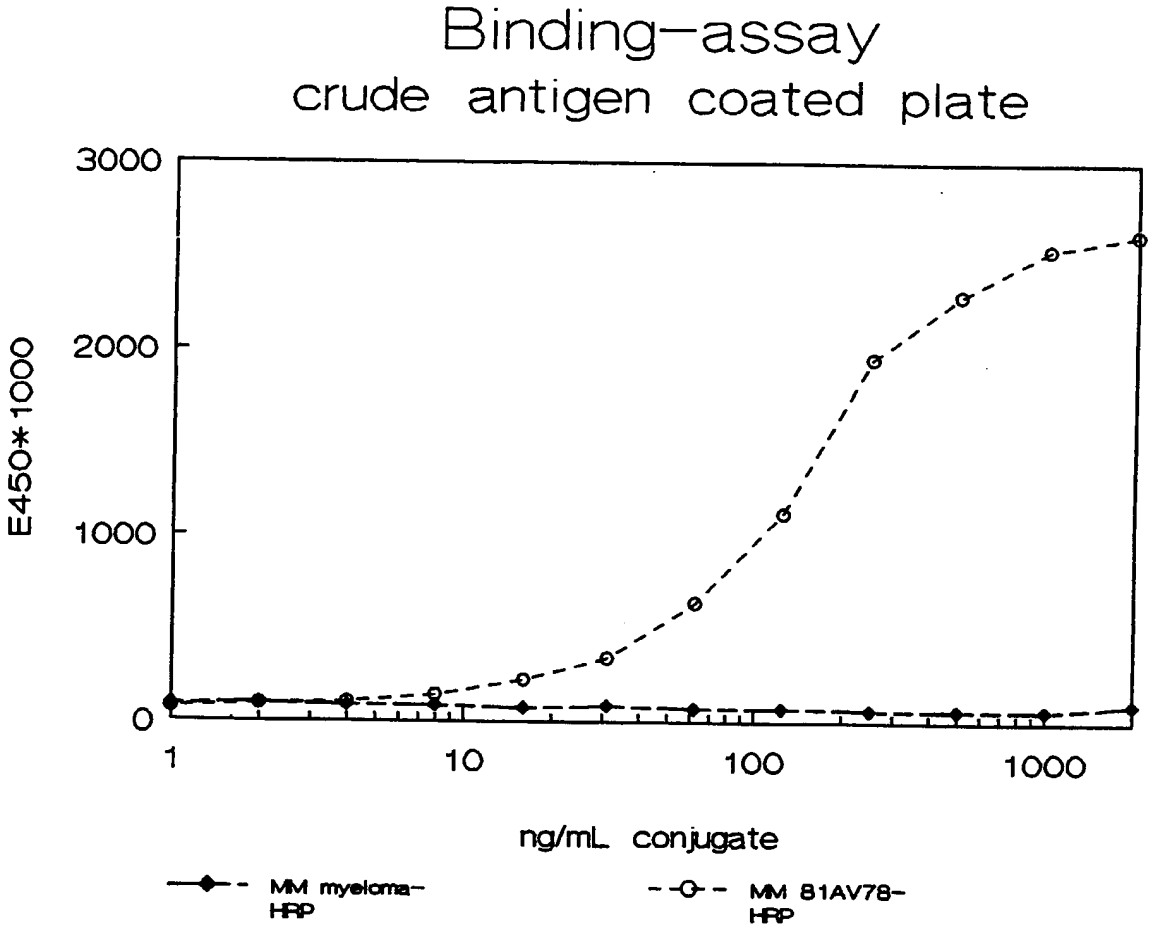
7. Immunoreactive compound according to claim 1-6, characterized in that to said polypeptide additionally are coupled one or more labels.

8. Immunoreactive compound according to claim 1-7, characterized in that said fragment is a monomer of IgM or IgA.

9. Immunoreactive compound according to claim 1-8, characterized in that said fragment is a $F(ab')_2$ fragment of IgM or IgA.

10. Therapeutically active composition containing an immunoreactive compound according to claim 1-9, which comprises at least one tumouricidic compound bound thereto.

11. Composition for diagnosis of cancer containing an immunoreactive compound according to claim 1-9, which comprises at least one diagnostically useful group bound thereto.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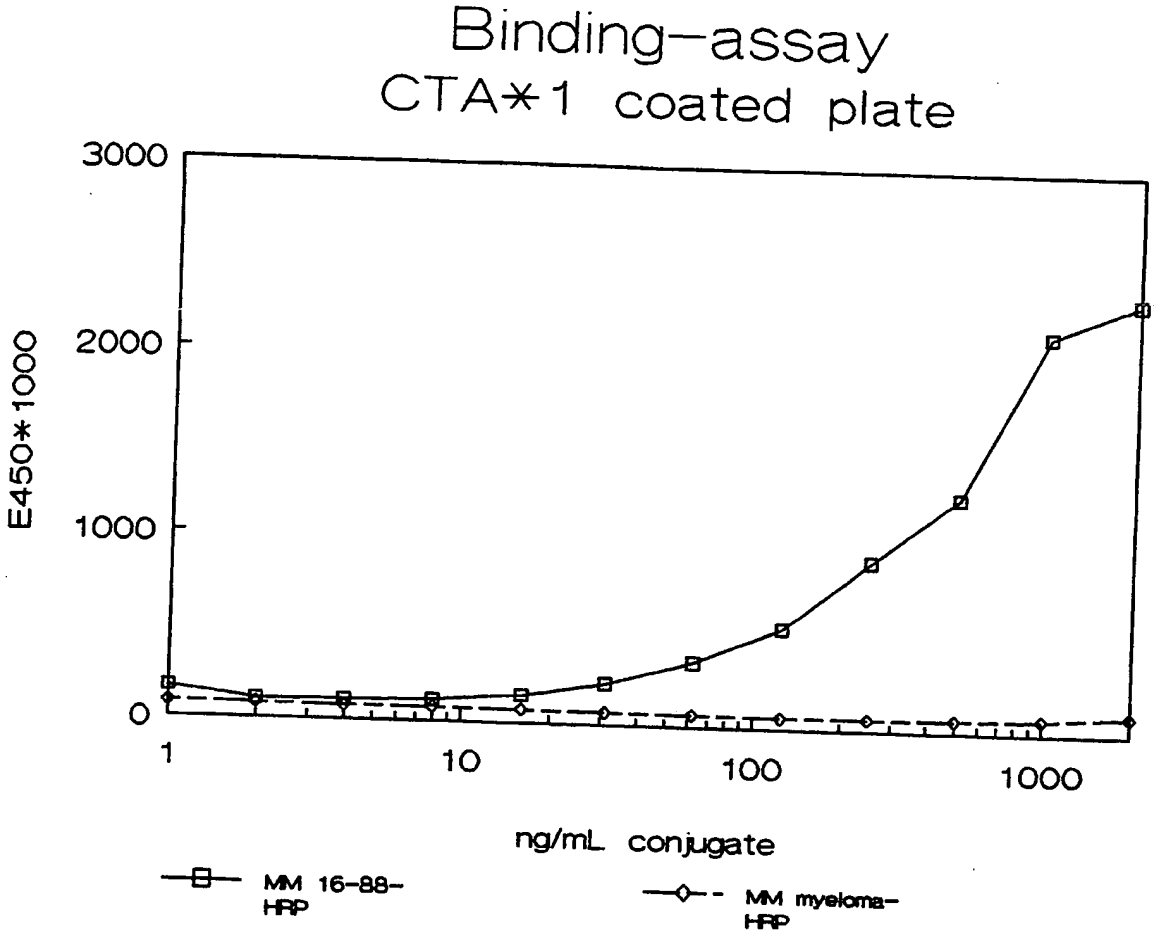
MM 81AV78–HRP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MM myeloma-HRP

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/01223

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 A 61 K 47/48 A 61 K 39/395 G 01 N 33/574
G 01 N 33/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	A 61 K G 01 N C 07 K C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
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X	EP,A,0303088 (MILES INC.) 15 February 1989, see column 3, lines 5-10; columns 3-4	1-11
X	CA,A,1168150 (M.J. POZNANSKY) 29 May 1984, see pages 4,25-28	1-11
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X	WO,A,8907649 (HIGHTECH RECEPTOR AB) 24 August 1989, see page 1, line 27 - page 2, line 12	1-2

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IV. CERTIFICATION

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International Searching Authority	**Signature of Authorized Officer**
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A	WO,A,8102522 (M.D. GOLDENBERG) 17 September 1981, see page 32, line 25	1-11
A	EP,A,0367166 (TAKEDA CHEMICAL INDUSTRIES) 9 May 1990, see column 6, line 32	1-11
A	EP,A,0151030 (LITTON BIONETICS) 7 August 1985, see the whole document (cited in the application)	1-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9101223 SA 48769

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9101223 SA 48769

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